OFFICIAL

1/31/02

#19

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:	)	Group Art Unit: 1644
VILEN et al.	)	Examiner: Roark, J.
Serial No.: 09/513,024	)	DECLARATION OF JOHN C. CAMBIER (Under 37 CFR 1.132)
Filed: February 25, 2000	)	
Atty. File No.: 2879-64	)	
For: "PRODUCT AND METHOD FOR TREATMENT OF CONDITIONS ASSOCIATED WITH RECEPTOR-DESENSITIZATION"	)	

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, John C. Cambier, declare as follows:

1. I am a co-inventor of the above-referenced patent application and am familiar with the application. I am a skilled artisan in the fields of immunology and molecular biology.

2. This Declaration is submitted in response to an Advisory Action having a mailing date of October 31, 2001 and in further response to an Office Action having a mailing date of August 8, 2001.

3. The following discussion is provided in response to the Examiner's rejection of Claims 1, 4-6, 9-10, 18-19, 21-22, 30-31 and 33 under 35 U.S.C. § 102(b) or § 103, with regard to the reference of Nakamura et al. (*Int. J. Hematol.* 64:39-46, 1996) (Nakamura A). Enclosed for the Examiner's review in connection with this discussion is a second reference by Nakamura et al. (*Int. Immunol.* 5(10):1309-1315, 1993) (Nakamura B).

The anti-CD79b (anti-Igβ) antibody that is described in Nakamura A and Nakamura B as "CB3-1" is distinguished from the antibody claimed in the present application by at least two characteristics. These characteristics are clearly ascertained from the publications by Nakamura et al. First, the CB3-1 antibody does <u>not</u> induce B cell unresponsiveness according to a classical assay

for B cell responsiveness. Second, the CB3-1 antibody stimulates the B cell antigen receptor. In contrast, the antibody recited in the claims of the present invention induces B cell unresponsiveness (i.e., by causing a dissociation or inhibiting an association between the B cell antigen receptor components) and does not stimulate the B cell antigen receptor.

B cell unresponsiveness

A classical assay by which B cell responsiveness (or conversely, unresponsiveness/anergy) is evaluated is the measurement of B cell proliferation and the expression of activation markers CD80 and CD86 as a result of antigenic stimulation (i.e., stimulation through the B cell antigen receptor). A responsive B cell will proliferate and upregulate CD80 and CD86 in response to antigenic stimulation. An unresponsive B cell, will not proliferate and will not upregulate CD80 and CD86 in response to antigen (i.e., the receptor is *desensitized* to antigen stimulation). In addition, it is known in the art that a desensitized B cell antigen receptor (i.e., expressed by an unresponsive B cell) does not elicit tyrosine phosphorylation or mobilize calcium in response to antigenic stimulation (or an appropriate mimic thereof), despite the continued expression of antigen receptors.

The classical assay for B cell responsiveness described above is precisely the assay that is used to measure B cell responsiveness in Nakamura A (see Section 3.3 and Figure 3). Referring to the paragraph bridging pages 42-43, Nakamura A states that the CB3-1 antibody did not inhibit the induction of CD80 and CD86 by stimulation of the B cell through the antigen receptor. Similarly, CB3-1 did not inhibit B cell proliferation of the B cell which was stimulated through the antigen receptor. One of skill in the art can conclude from this assay that the CB3-1 antibody does not induce B cell unresponsiveness as defined above, and indeed, that is what Nakamura and colleagues have concluded (see page 43, 1st column, last sentence; and page 45, 2nd column, first sentence).

Turning to the present application, Dr. Vilen and I have discovered that the extracellular ligand binding component of the B cell receptor (mIg) can be physically uncoupled from its associated transducer, and that this dissociation from mIg mediates the destabilization of the B cell receptor in B cells and thus mediates the unresponsive state (see Examples 1-8). In other words, we have discovered that the dissociation of the receptor components is the mechanism by which B cell unresponsiveness resulting from receptor destabilization occurs.

Given these teachings regarding the mechanism for B cell unresponsiveness, one of skill in the art can conclude that the antibody of Nakamura A does not cause dissociation of or inhibit an association between the B cell antigen receptor components effective to induce B cell unresponsiveness, because this antibody did not cause B cell unresponsiveness according to art-recognized, classical assays.

In contrast to the antibody of Nakamura et al., Dr. Vilen and I have produced an antibody that takes advantage of the mechanism described above by binding to the extracellular domain of the transducer component and inducing the unresponsive state in B cells (i.e., the antibody causes a dissociation of or inhibits an association between the B cell antigen receptor components). For example, we have shown that the antibody of the present invention significantly inhibits calcium mobilization in B cells that have been stimulated with antigen (Example 9 and Figure 8), showing that this antibody induces B cell unresponsiveness according to an art-accepted assay for B cell activation.

B cell stimulation

Referring to Nakamura B, this reference demonstrates that the CB3-1 antibody stimulates the B cell, which shows that the antibody of Nakamura A and B does not meet the limitations of the present claims, and which additionally shows that the antibody of Nakamura would not be useful in a therapeutic application (i.e., any stimulation of the B cell response is undesirable in an immunosuppressive application). Specifically, Figure 2 of Nakamura B shows that the CB3-1 antibody induces significant phosphatidylinositol hydrolysis in B cells, indicating that this antibody stimulates the B cell antigen receptor. Figure 3 of Nakamura B shows that the CB3-1 antibody induces a significant increase in intracellular calcium in B cells, again indicating that this antibody stimulates the B cell antigen receptor. The authors state at page 1311, 1st column, last sentence, that these "results suggest that anti-Igβ mAbs are functionally capable of triggering early B cell activation events." In addition, it is noted that the CB3-1 antibody stimulated B cell proliferation (Figure 4), again indicating that this antibody stimulates B cell activation through the B cell antigen receptor.

In contrast, the antibody of the present invention does not significantly stimulate the B cell antigen receptor, as demonstrated by a calcium mobilization assay (Example 9 and Figure 8).

5. I hereby declare that all statements made herein of my own are true and that all statements made on information and belief are believed to be true; and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing therefrom.

Date: 1/31/02

By: [signature]
John C. Cambier

International Immunology, Vol. 5, No. 10, [redacted]309 – 1315

Signal transduction in human B cells initiated via Igβ ligation

Tetsuya Nakamura[1], M. Chandra Sekar, Hiromi Kubagawa, and Max D. Cooper

Division of Developmental and Clinical Immunology, Departments of Medicine, Pathology, Pediatrics, and Microbiology, University of Alabama at Birmingham, and the Howard Hughes Medical Institute, Birmingham, AL 35294, USA

[1]Present address: First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

Key words: B cell activation, Igα, mb-1, surface Ig

Abstract

Igα and Igβ heterodimers are non-covalently associated with Ig to compose the antigen receptor complexes on B cells. The demonstration that different sets of tyrosine kinases bind to the cytoplasmic tails of Igα and Igβ suggests that Igα and Igβ may activate distinct second messenger pathways. In this study, we examined the effects of mAbs against an exposed epitope of human Igβ on pre-B and B cell triggering. Cross-linkage of Igβ on B cells leads to activation of tyrosine kinases, hydrolysis of phosphatidylinositides, and elevation of intracellular Ca^{2+}, effects qualitatively identical to those of anti-μ mAbs. Our observations thus indicate that cross-linking of Igβ does not segregate signal transduction pathways connected with the cytoplasmic tails of Igα and Igβ. Igα ligation has been reported to be more effective in triggering pre-B than B cells, whereas our results indicated that Igβ ligation is more efficient in triggering B than pre-B cells. In addition to their activation properties, the anti-Igβ mAbs effectively modulated B cell receptor complexes and blocked terminal differentiation of all plasma cell isotypes. The findings support the idea that anti-Igβ could serve as a universal B cell immunosuppressant.

Introduction

Surface Igs on B cells are physically linked to the mb-1 gene product, Igα (1), and the B29 gene product, Igβ (2). These molecules form the B cell antigen receptor (BCR) complex. The cytoplasmic signals initiated by antigen binding to surface Ig are delivered through the Igα and Igβ transmembrane molecules (3,4). Recent studies indicate that the cytoplasmic tail of Igα is physically linked to *src* family tyrosine kinases *lyn* and *fyn*, and phosphatidylinositol-3 kinase, whereas Igβ is linked to phosphatidylinositol-3 kinase and other unidentified phosphoproteins (5). Tyrosine kinases *blk* and *lck* are also associated with the BCR (6 – 10). These tyrosine kinases are activated by BCR ligation to phosphorylate a variety of cellular proteins, including phospholipase C (γ1 and γ2) (11 – 13), GTPase activating protein (GAP) (14), and microtubule-associated protein-2 kinase (MAP-2K) (15). The phosphorylation of phospholipase C and GAP is involved in phosphoinositide (PI) hydrolysis, the subsequent activation of protein kinase C and elevation of intracellular Ca^{2+} (16). MAP-2K phosphorylates serine residues on the c-*jun* transcription factor that may relay BCR-mediated signals to the nucleus (17). These known tyrosine kinase substrates together with other unknown substrates comprise complex signal transduction pathways which may link BCR signaling to nuclear activation events.

As one approach to investigation of the Igα and Igβ roles in this complex signal transduction cascade, we have generated mAbs against an exposed epitope of the human Igβ chain (18). In the present study these mAbs were compared with anti-μ heavy chain (HC) mAbs to determine their effects on early B cell activation events and on terminal plasma cell differentiation. The results indicate that both anti-Igβ and anti-μ mAbs induce quantitatively different, but qualitatively similar B cell signals, and that anti-Igβ mAbs can inhibit terminal differentiation of plasma cells regardless of their Ig isotype.

Methods

Antibodies and cells

Mouse mAbs to human μ (SA-DA-4.4 and 145-8, $\gamma1\kappa$ isotype), α (CH-EB6, $\gamma1\kappa$), κ (TB28-2, $\gamma1\kappa$), and λ (1-155-2, $\gamma1\kappa$) chains,

Correspondence to: M. D. Cooper, 278 Wallace Tumor Institute, UAB station, Birmingham, AL 35233, USA

Transmitting editor: T. Takemori

Received 28 April 1993, *accepted* 12 July 1993

and Igβ (CB3-1 and CB3-2, γ1κ) and a rat antibody to mouse κ (187-1) were described previously (18–22). Monoclonal anti-CD19 antibody was purchased from Becton-Dickinson (Mountain View, CA) and monoclonal anti-phosphotyrosine (4G10) antibody was from UBI (Lake Placid, NY). Monoclonal anti-human γ antibody, affinity-purified goat antibodies specific for human light chains labeled with fluorescein isothiocyanate, goat antibodies to mouse Ig labeled with peroxidase, and alkaline phosphatase-labeled streptavidin were from Southern Biotechnology Associates (Birmingham, AL). The Ramos B cell line expressing surface IgM and the 697 pre-B cell line expressing μ HC and surrogate light chains were described previously (23).

Immunoblotting

Ramos B cells or 697 pre-B cells washed with PBS were resuspended in RPMI 1640 medium supplemented with 10% FCS (HyClone, Logan, UT) and 20 mM HEPES (pH 7.4), and 100 μl aliquots of cell suspension (10^7/ml) were warmed at 37°C for 10 min. Stimulation was initiated by adding each mAb at a defined concentration for 5 min at 37°C and the reaction was stopped by adding 1 ml of ice-cold PBS with 1 mM Na_3VO_4. Cells were centrifuged and lysed in 40 μl of the lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.1% trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride). The lysates were resolved by SDS–10% PAGE under reducing conditions and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Phosphoproteins were detected by anti-phosphotyrosine mAb (4G10), followed by peroxidase-labeled goat anti-mouse Ig antibody and the enhanced chemiluminescence system (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Measurement of PI hydrolysis

Ramos B cells washed twice with PBS were resuspended in Media 199 (Gibco BRL, Grand Island, NY) supplemented with 10% dialyzed FCS at 3×10^6/ml. After myo-[2-^{3}H]inositol (Amersham) was added at a final concentration of 10 μCi/ml, the cells were incubated for 4 h at 37°C. After twice washing with PBS, cells (5×10^6/ml) were resuspended in Media 199. Cells (200 μl aliquots) were kept on ice for 30 min and incubated with 2 μl of 1 M LiCl for 15 min at 37°C before stimulation with the test mAbs (50 μg/ml final concentration) for 30 min at 37°C. Isotype matched mAb (γ1κ) with irrelevant specificity served as negative control. To extract hydrolyzed PI, cells were centrifuged at 15,600 *g* for 10 s, resuspended in 800 μl of boiling water, and immersed in boiling water for 5 min. After centrifugation for 5 min at 15,600 *g*, the supernatants were collected and applied on a SAX column (Whatmann, Hillsboro, OR). PI were separated by HPLC by the following elution profile: 2 min with water; 20 min with a linear gradient of 0–20% 1 M ammonium formate (pH 3.75); 25 min with a linear gradient of 20–100% 1 M ammonium formate. Fractions were counted by liquid scintillation spectroscopy and total c.p.m. of fractions corresponding to inositol monophosphate was determined.

Determination of intracellular Ca^{2+}

Ramos B cells or 697 pre-B cells were resuspended in the loading buffer (Hanks' balanced salt solution containing 1.3 mM $CaCl_2$) at 5×10^6/ml. Fluo-3 (Molecular Probes, Eugene, OR) was added at the final concentration of 3 μM and cells were incubated for 30 min at 37°C. After twice washing with PBS, cells were resuspended in the loading buffer at 4×10^5/ml and fluorescence intensity was analyzed on a FACScan (Becton-Dickinson) with 488 nm excitation and 525 nm measurement. The mAbs (20 μg/ml final concentration) were added 1 min after the start of analysis and the cumulative fluorescence was determined for an additional 5 min. Results were plotted by time course versus relative fluorescence using linear amplification.

Proliferation assay

Mononuclear cells (MNCs) were prepared from peripheral blood of healthy volunteers by centrifugation over Ficoll-Hypaque density gradient and B cells were enriched by removing the E rosette-forming cells. B-enriched MNC (5×10^5) or Ramos B cells (5×10^4) were resuspended in RPMI medium with 10% FCS and incubated with various concentrations of mAbs in flat-bottomed wells for 3 days (200 μl/well). In some experiments, Sepharose 4B beads coupled with rat anti-mouse κ mAb (2 mg mAb/ml of Sepharose 4B) were added in a final 1% suspension. At 16 h before harvest, 1 μCi of [^{3}H]thymidine (Amersham) was added. Triplicate cultures were analyzed and the results expressed as mean c.p.m.

Modulation assay

Blood MNCs (2×10^6/ml) were cultured with various mAb concentrations for 16 h at 37°C. Cells were then washed and stained with FITC-labeled goat antibodies to human Ig light chains. The B cells were counter-stained with phycoerythrin-labeled anti-CD19 mAb and the mean fluorescence intensity (MFI) determined for B cell expression of light chains with and without prior antibody treatment. Specific MFI was calculated by subtracting MFI of autofluorescence from MFI of samples and percent expression of BCR was represented as follows:

$$\frac{\text{specific MFI with antibody treatment}}{\text{specific MFI without antibody treatment}} \times 100.$$

B cell differentiation assay

Blood MNC (10^6/ml) were resuspended in RPMI medium with 10% FCS and cultured with 1:100 dilution of pokeweed mitogen (PWM; Gibco BRL, Gaithersburg, MD) in the presence of test mAbs (10 μg/ml) for 10 days in flat-bottomed 96-well plates (200 μl/well). After 10 days culture, secreted Igs in the supernatants were measured by ELISA to measure secreted Ig. Supernatants were added to plastic wells coated with either anti-human γ or μ (145-8) mAb, and the bound Igs were detected by a mixture of biotinylated anti-human κ and λ mAbs, followed by alkaline phosphatase-labeled streptavidin and substrates. Results are represented as the mean OD_{405} of triplicate cultures.

Results

Early activation events induced by anti-Igβ mAbs

We first examined whether the anti-Igβ mAbs can activate BCR signal transduction pathways that initiate early activation events: tyrosine phosphorylation, PI hydrolysis, and elevation of intracellular Ca^{2+} (14,16). Incubation with either anti-Igβ (CB3-1 and CB3-2)

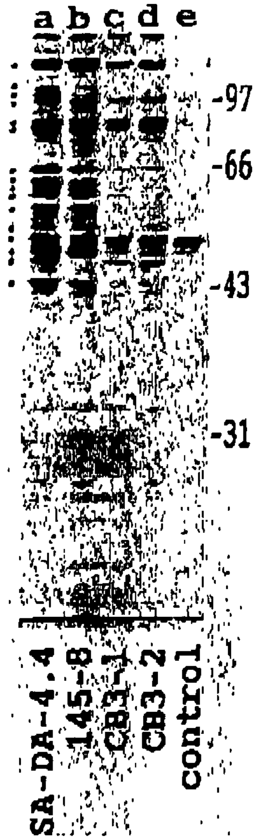

Fig. 1. Protein tyrosine phosphorylation induced by anti-Igβ and anti-μ mAbs. Ramos B cells (10^8) were stimulated with SA-DA-4.4 (a), 145-8 (b), CB3-1 (c), CB3-2 (d), and isotype-matched control mAb (e). Cell lysates were resolved by SDS – PAGE, transferred to a PVDF membrane, and phosphotyrosine was detected by an anti-phosphotyrosine mAb. Phosphorylated proteins are marked with dots. These results, except for altered migration of a 50 kDa phosphoprotein in lane b, were reproduced in two additional experiments.

of the anti-μ mAbs (SA-DA-4.4 and 145-8) induced or upregulated phosphorylation of tyrosine residues on at least 16 proteins in Ramos B cells (marked with dots in Fig. 1). While induced phosphorylation intensity differed for each mAb, the pattern of phosphorylated proteins was essentially identical. SA-DA-4.4 was the most potent stimulator, 145-8 was intermediate, and CB3-1 and CB3-2 gave the weakest signals. Next, we examined whether anti-Igβ mAbs could induce PI hydrolysis and elevate intracellular Ca^{2+} levels. Ramos B cells which had incorporated myo-[2-^{3}H]inositol were stimulated with anti-Igβ and anti-μ mAbs, and the hydrolyzed inositol monophosphate was measured as a marker of PI hydrolysis. Although both anti-Igβ mAbs induced an increase of inositol monophosphate, the anti-μ mAbs were stronger stimulants than the anti-Igβ mAbs (Fig. 2). Similarly, the anti-Igβ mAbs induced smaller and relatively delayed elevations of intracellular Ca^{2+} in Ramos B cells by comparison with the more efficeint anti-μ mAb (Fig. 3). These results suggest that anti-Igβ mAbs are functionally capable of triggering early B cell activation events, albeit less efficiently than the two anti-μ mAbs: SA-DA-4.4 > 145-8 > CB3-1 = CB3-2.

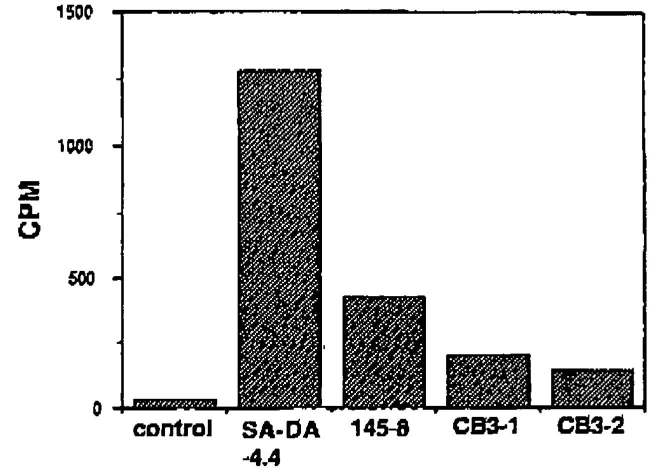

Fig. 2. PI hydrolysis induced by anti-Igβ and anti-μ mAbs. Ramos B cells (10^6) were stimulated with 50 μg/ml of isotype-matched control mAb, SA-DA-4.4, CB3-1 or CB3-2 antibodies for 30 min in the presence of LiCl. Hydrolyzed PI was extracted and separated by HPLC. Total c.p.m. corresponding to inositol monophosphate are represented.

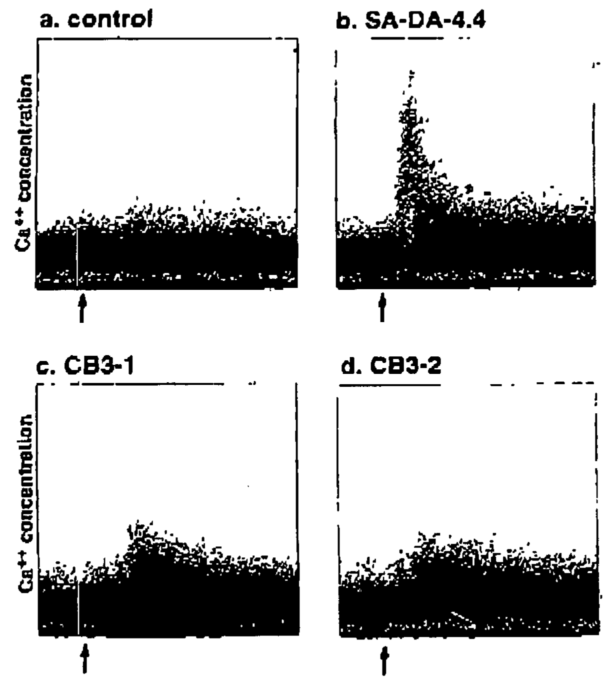

Fig. 3. Elevation of intracellular Ca^{2+} induced by anti-Igβ and anti-μ mAbs. Ramos B cells loaded with 3 μM Fluo-3 were analyzed on a FACScan. An isotype matched control mAb (a), SA-DA-4.4 (b), Cb3-1 (c), and CB3-2 (d) (20 μg/ml) were added 1 min after the start of analysis (indicated by arrows), and fluorescence intensity representing relative intracellular Ca^{2+} concentration measured over the next 5 min.

Late cellular response initiated by anti-Igβ mAbs

With the knowledge that both anti-Igβ mAb are functionally active, we examined whether the cross-linking of Igβ could induce a late

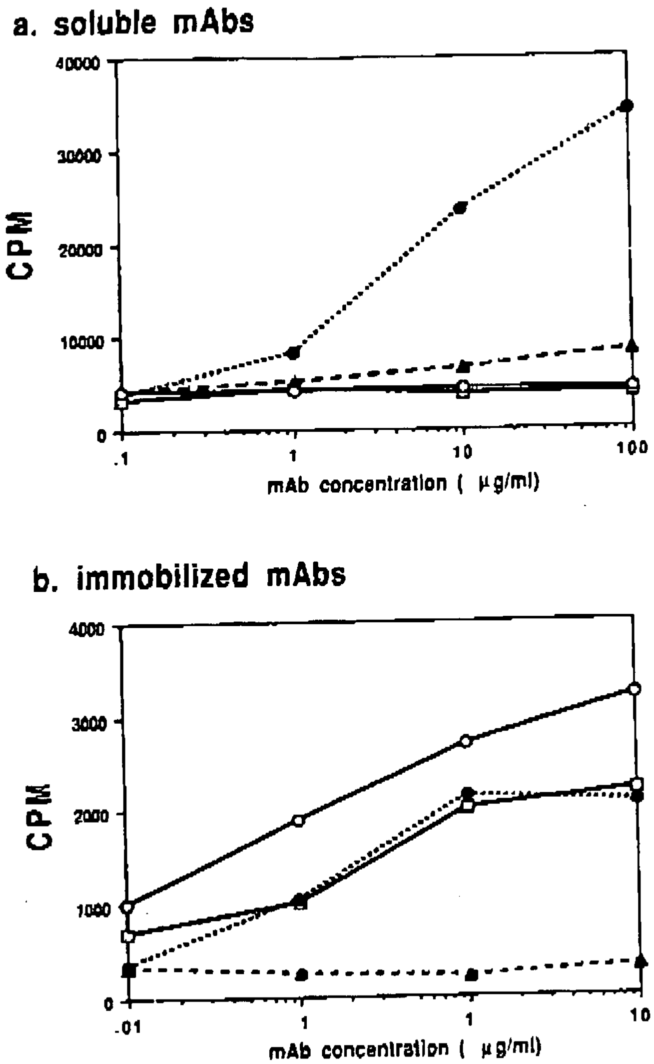

Fig. 4. Proliferation of B cells stimulated by anti-Igβ and anti-μ mAbs. E⁻ peripheral blood MNCs (2×10^5) were incubated with SA-DA-4.4 (····●····), CB3-1 (—O—), CB3-2 (—□—), or an isotype-matched control antibody (—★—) in various concentrations for 3 days before the incorporation of [^{3}H]thymidine was measured. Antibodies were used either in a soluble form (a) or immobilized to Sepharose 4B beads coated with rat anti-mouse κ mAb (b).

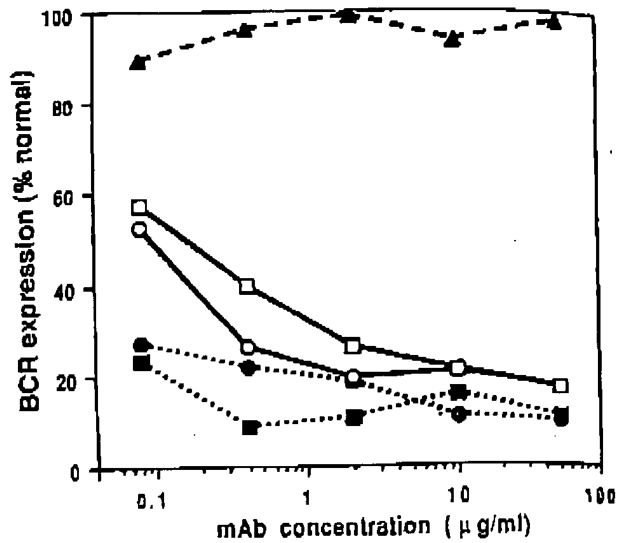

Fig. 5. Modulation of BCR by anti-Igβ and anti-μ mAbs. Blood B cells (~2×10^6/ml) were cultured either with 145-8 (····■····), SA-DA-4.4 (····●····), CB3-1 (—O—), CB3-2 (—□—), isotype-matched control mAb (—★—), or without antibody for 16 h at 37°C. B cell light chain expression was analyzed by cell surface immunofluorescence and the percent of normal BCR expression calculated as described in Methods

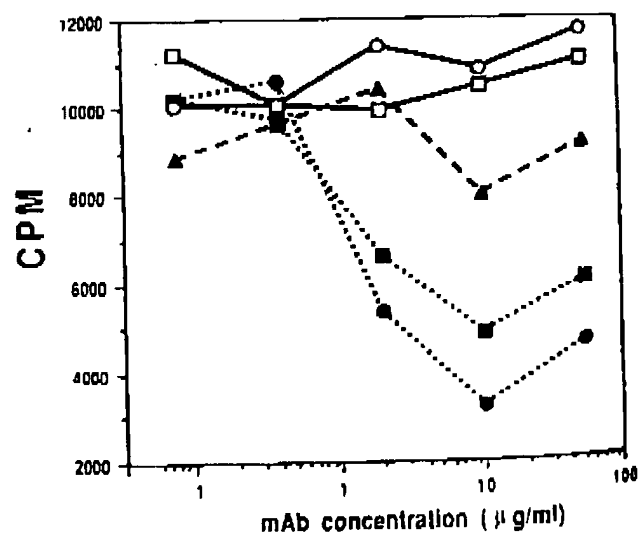

Fig. 6. Effects of anti-Igβ and anti-μ mAb on Ramos B cell growth. Ramos B cells (5×10^4/ml) were incubated with 145-8 (····■····), SA-DA-4.4 (····●····), CB3-1 (—O—), CB3-2 (—□—), or an isotype-matched control mAb (—★—) in various concentrations for 3 days. Incorporation of [^{3}H]thymidine was measured and the mean values of triplicate cultures presented

cellular response measured as the induction of DNA synthesis by normal B cells, modulation of BCR on B cells, and growth inhibition of Ramos B cells. When peripheral B cell preparations were incubated for 3 days with anti-Igβ or anti-μ mAbs in soluble form, the anti-μ mAbs induced proliferation but the anti-Igβ did not (Fig. 4a). However, when these mAbs were immobilized to Sepharose 4B beads coupled with rat anti-mouse κ mAb, all of the mAbs had strong proliferative effects on peripheral B cells (Fig. 4b). This suggests that in soluble form the anti-Igβ mAbs do not achieve sufficient cross-linking to induce DNA synthesis but this inadequacy can be overcome by immobilizing the CB3 antibodies on a solid matrix.

In contrast to the above results, the anti-Igβ mAb in soluble form could efficiently down-modulate the cell surface expression of BCR. When B cells from peripheral blood were cultured with various concentrations of anti-Igβ and anti-μ mAbs and the expression of light chains on CD19⁺ B cells analyzed after 16 h,

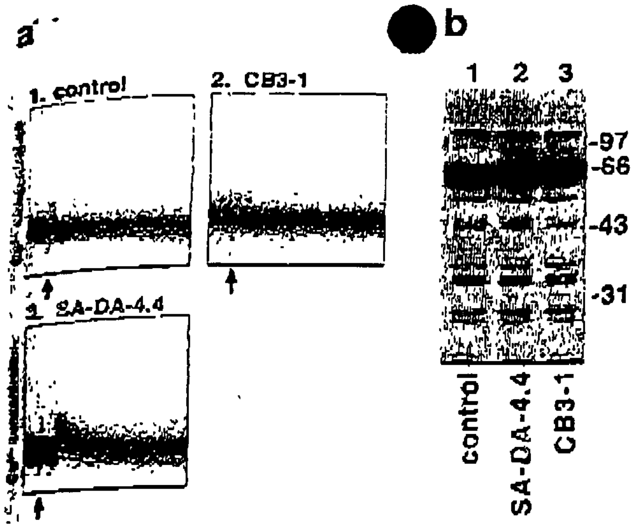

Fig. 7. Early activation effects of anti-Igβ and anti-μ mAbs on pre-B cells. (a) 697 pre-B cells loaded with 3 μm Fluo-3 were analyzed on a FACScan. An isotype matched control mAb (1), CB3-1 (2), and SA-DA-4.4 (3) (20 μg/ml) were added 1 min after the start of the analysis (indicated by arrows) and fluorescence intensity representing relative intracellular Ca^{2+} concentration measured over the next 5 min. (b) 697 pre-B cells (10⁶) were stimulated with an isotype-matched control mAb (1), SA-DA-4.4 (2), and CB3-1 (3). Cell lysates were resolved by SDS – PAGE, transferred to a PVDF membrane and phosphotyrosine was detected by an anti-phosphotyrosine mAb.

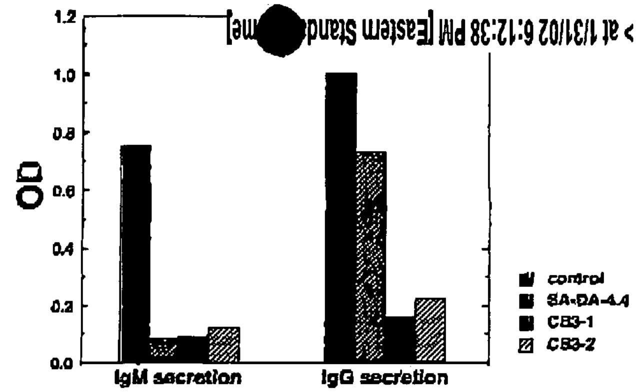

Fig. 8. Inhibition of terminal plasma cell differentiation by anti-Igβ and anti-μ mAbs. Peripheral MNCs (10^6/ml) were cultured with 10 μg/ml of an isotype-matched control mAb, SA-DA-4.4, CB3-1, or CB3-2 for 10 days in the presence of PWM. Igs secreted into supernatants were measured by ELISA as described in Methods.

all of the mAbs were effective modulators, although higher concentrations of anti-Igβ mAbs were required than for the anti-μ (Fig. 5). Finally, when the antibody effects on growth of Ramos B cells was examined, neither of the anti-Igβ mAbs inhibited cell growth of B cells, whereas both anti-μ mAbs were inhibitory (Fig. 6).

The effects of anti-Igβ and anti-μ mAbs on 697 pre-B cells

Since cross-linkage of Igα in mice has been reported to be more effective in pre-B cells than mature B cells (24), it was of interest to test the effect of cross-linkage of Igβ on pre-B cells. For this purpose, we used 697 pre-B cells expressing μ HC, surrogate light chains, Igα and Igβ on the cell surface and examined whether anti-Igβ mAbs induce early activation events in this cell line. As shown in Fig. 7(a), the anti-μ mAb induced the elevation of intracellular Ca^{2+}, whereas neither anti-Igβ mAbs exhibited detectable effects. We next tested tyrosine phosphorylation of cellular proteins by anti-Igβ mAbs in 697 pre-B cells. Neither the anti-μ nor anti-Igβ mAbs induced any detectable increase in tyrosine phosphorylation (Fig. 7).

Inhibition of terminal B cell differentiation by anti-Igβ mAbs

Isotype-specific antibodies have been shown to inhibit the terminal differentiation of human B cells in a PWM-driven system (19,25). Assuming that anti-Igβ ligation provides signals similar to those by anti-μ ligation, the CB3 mAbs should also prevent B cell differentiation. To test this hypothesis, MNC from peripheral blood were cultured for 10 days with the different BCR mAbs in the presence of PWM, and the Ig secreted into the supernatants were measured. As shown in Fig. 8, both of the anti-Igβ CB3 mAbs inhibited the secretion of IgM and IgG, whereas the anti-μ mAb primarily inhibited IgM secretion as reported previously (19,25).

Discussion

In these studies, the CB3-1 and CB3-2 antibodies, which are directed against an exposed epitope of human Igβ, were found to be capable of activating tyrosine kinases, inducing PI hydrolysis, and initiating an elevation of intracellular Ca^{2+} levels, key metabolic activities in the major signal transduction pathways triggered via the BCR (3,14,16). Clark *et al.* (5) have shown that the cytoplasmic tails of Igα and Igβ bind to different tyrosine kinases to activate distinct second messenger pathways. Both the anti-μ induced BCR modulation and subsequent growth inhibition of B cell lines are dependent on tyrosine kinase activities (26,27), but tyrosine kinase activation *per se* does not necessarily lead to B cell proliferation (28). It seemed possible, therefore, that the cross-linking effects of the anti-Igβ and anti-μ mAbs would activate different sets of tyrosine kinases and result in different patterns for the late cellular responses. However, this idea is not supported by our results. Both anti-Igβ and the anti-μ mAbs induced phosphorylation of tyrosine residues on the same protein substrates. This suggests that both types of mAbs can activate the same second messenger pathways involving regulation by phosphorylation of tyrosine residues. In our evaluation of anti-μ and anti-Igβ ligation in the late cellular response, the anti-Igβ mAbs had no demonstrable effect on B cell proliferation nor did they inhibit the growth of the Ramos B cells, whereas the anti-μ mAbs were effective in both respects. However, this seems unlikely to mean that anti-μ mAbs deliver a qualitatively different signal from that of anti-Igβ mAbs, but more likely that each response requires a different minimum level of stimulation to elicit the response. In this view the anti-Igβ mAbs, being the weakest stimulators, do not exceed the necessary threshold. This explanation is supported by the observation that, when immobilized on the surface of Sepharose beads, the anti-Igβ mAbs efficiently induced B cell proliferation. These results therefore suggest that the cross-linkage of Igβ or μ HC provides qualitatively identical

signals, probably because the cross-linkage of Igβ alters the conformational relationships of not only Igβ but also the covalently-linked Igα chain to activate the tyrosine kinases that bind to both Igα and Igβ.

Studies by Nomura *et al.* (24) suggest that cross-linkage of Igα in mice initiates signal transduction for pre-B cells but not for mature B cells, whereas μ HC cross-linkage is effective essentially in activation of mature B cells (24). On the contrary, we found that both anti-Igβ mAbs and anti-μ mAbs activate signal transduction by mature B cells from the circulation and the Ramos B cell line. When the effect of anti-Igβ mAbs was tested on a pre-B cell line 697 expressing μ HC, surrogate light chain, and Igα and Igβ in the cell surface (18), cross-linkage of Igβ did not induce detectable tyrosine phosphorylation or elevation of intracellular Ca^{2+}, whereas that of μ HC caused elevation of intracellular Ca^{2+}. This difference between cross-linking of Igα and Igβ may suggest variation in the functional predominance of Igα and Igβ as a function of B cell differentiation. The following observations could support this idea. First, surface immunofluorescence analysis revealed that the expression level of Igβ and μ HC is linearly correlated in both human (18) and mouse B (29) lineage cells, suggesting a constant molecular ratio of Igβ versus μ HC throughout B cell differentiation. However, it has been reported that the expression level of Igα is relatively constant regardless of the variable expression level of μ HC and that a pre-B cell line that does not yet express detectable surface μ HC is already surface Igα positive (28,30). Secondly, Mason *et al.* (31) recently showed that out of 25 cases of acute lymphoblastic leukemia most of the cases expressed cytoplasmic Igα, whereas only a half of them expressed Igβ, suggesting that the cytoplasmic expression of Igα precedes that of Igβ. The latter two observations may indicate that Igα is expressed more predominantly than Igβ on early B cells. Since this could have significant functional implications, it will be important to test this idea.

The anti-Igβ mAbs exhibit pan-B cell inhibitory effects via their ability to modulate BCR associated with all isotypes of Igs (IgM, IgD, IgG, and IgA; our unpublished observations), and to inhibit plasma cell differentiation. These *in vitro* properties support the idea that the anti-Igβ antibodies could serve as universal B cell suppressors in clinical situations (18). This hypothesis presumes that *in vivo* exposure of B cells to anti-Igβ mAbs would modulate the BCR to prevent antigen recognition and that terminal B cell differentiation would be inhibited by anti-Igβ treatment. If these predictions hold true, the anti-Igβ mAbs theoretically would be superior to anti-sIg antibodies for targeting B cells *in vivo*, because the former would not encounter their target cellular antigen in the form of soluble products in the circulation.

Acknowledgements

We thank Ms Jutta Jurgen for technical assistance. This work was supported in part by AI30879, AI23694, and CA13148 awarded by the National Institutes of Health. M. D. C is an Investigator of the Howard Hughes Medical Institute.

Abbreviations

BCR	B cell receptor
GAP	GTPase activating protein
HC	heavy chain
MAP-2K	microtubule-associated protein-2 kinase
MFI	mean fluorescence intensity
MNC	mononuclear cell
PI	phosphatidylinositide
PWM	pokeweed mitogen

References

1 Sakaguchi, N., Kashiwamura, S., Kimoto, M., Thalmann, P., and Melchers, F 1988. B lymphocyte lineage restricted expression of mb-1, a gene with CD3-like structure properties. *EMBO J* 7:3457.
2 Hermanson, G. G., Eisenberg, D., Kincade, P. W , and Wall, R. 1988. A member of the immunoglobulin gene superfamily exclusively expressed on B-lineage cells. *Proc. Natl Acad. Sci USA* 85:6890.
3 Reth, M. 1992. Antigen receptors on B lymphocytes. *Annu, Rev. Immunol.* 10:97.
4 Costa, T. E., Franke, R. R., Sanchez, M., Misulovin, Z., and Nussenzweig, M. C. 1992. Functional reconstitution of an Immunoglobulin antigen receptor in T cells. *J Exp. Med.* 175:1669.
5 Clark, M. R., Campbell, K. S., Kazlauskas, A., Johnson, A. A., Hertz, M., Potter, T. A., Pleiman, C , and Cambier, J. C. 1992. The B cell antigen receptor complex: association of Ig-α and Ig-β with distinct cytoplasmic effectors. *Science* 258:123.
6 Gold, M. R., Law, D. A., and DeFranco, A. L 1990. Stimulation of protein tyrosine phosphorylation by the B-lymphocyte antigen receptor. *Nature* 345:810.
7 Gold, M. R., Matsuhashi, L., Kelly, R. B., and DeFranco, A. L 1991. Tyrosine phosphorylation of components of the B-cell antigen receptors following receptor crosslinking. *Proc. Natl Acad. Sci. USA* 88:3346.
8 Burkhardt, A. L., Brunswick, M., Bolen, J . B., and Mond, J. J. 1991. Anti-immunoglobulin stimulation of B lymphocytes activates src-family protein-tyrosine kinases. *Proc. Natl Acad. Sci. USA* 88.7410.
9 Yamanashi, Y , Fukui, Y., Wongsasany, B., Kinoshita, Y., Ichimori, Y., Toyoshima, K., and Yamamoto, T. 1992. Activation of Src-like protein-tyrosine kinase Lyn and its association with phosphatidylinositol 3-kinase upon B-cell antigen receptor-mediated signalling. *Proc. Natl Acad. Sci. USA* 89:1118.
10 Lin, J. and Justement, L. B. 1992. The MB-1/B29 heterodimer couples the B cell antigen receptor to multiple *src* family tyrosine kinases. *J. Immunol.* 149:1548.
11 Hempel, W. M., Schatzman, R. C., and DeFranco, A. L 1992. Tyrosine phosphorylation of phospholipase C γ2 upon crosslinking of membrane Ig on mature B lymphocytes. *J. Immunol.* 148:3021.
12 Coggeshall, K. M., McHugh, J. C., and Altman, A. 1992. Predominant expression and activation-induced tyrosine phosphorylatation of phospholipase C-γ2 in B lymphocytes. *Proc. Natl Acad. Sci. USA* 89:5660
13 Carter, R. H., Park, D .J., Rhee, S. G , and Fearon, D. T. 1991. Tyrosine phosphorylation of phospholipase C induced by membrane immunoglobulin in B lymphocytes *Proc. Natl Acad. Sci. USA* 88:2745.
14 Cambier, J. C. and Campbell, K. S. 1992. Membrane immunoglobulin and its accomplices: new lessons from an old receptor. *FASEB J.* 6.3207.
15 Casillas, A., Hanekom, C., Williams, K., Katz, R., and Nel, A. E. 1991. Stimulation of B-cells via the membrane immunoglobulin receptor or with phorbol myristate 13-acetate induces tyrosine phosphorylation and activation of a 42-kDa microtubule associated protein-2 kinase. *J. Biol. Chem.* 266:19088.
16 Sefton, B. M. and Campbell, M. 1991. The role of tyrosine protein phosphorylation in lymphocyte activation. *Annu Rev. Cell. Biol.* 7:257.
17 Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. 1991. Phosphorylation of c-*jun* mediated by MAP kinase. *Nature* 353:670.
18 Nakamura, T., Kubagawa, H., and Cooper, M. D. 1992. Heterogeneity of immunoglobulin-associated molecules on human B cells identified by monclonal antibodies. *Proc. Natl Acad. Sci. USA* 89:8522.
19 Maruyama, S., Kubagawa, H., and Cooper, M. D. 1985. Activation of human B cells and inhibition of their terminal differentiation by monoclonal anti-μ antibodies. *J. Immunol.* 135:192.
20 Kubagawa, H., Gathings, W. E., Levitt, D., Kearney, J. F., and Cooper, M. D. 1982. Immunoglobulin isotype expression of normal pre-B cells as determined by immunofluorescence. *J. Clin. Immunol.* 2:264.

21 Kiyotaki, M., Cooper, M. D., Bertoli, L. F., Kearney, J. F., and Kubagawa, H. 1987. Monoclonal anti-Id antibodies react with varying proportions of human B lineage cells. *J. Immunol.* 132:4150.

22 Yelton, D. E., Desaymard, C., and Scharff, M. D. 1981. Use of monoclonal anti-mouse immunoglobulin to detect mouse antibodies. *Hybridoma* 1:5.

23 Ha, H., Kubagawa, H., and Burrows, D. P. 1992. Molecular cloning and expression pattern of a human gene homologous to the murine mb-1 gene. *J. Immunol.* 148:1526.

24 Nomura, J., Matsuo, T., Kubota, E., Kimoto, M., and Sakaguchi, N. 1991. Signal transduction through the B cell-specific MB-1 molecule at the pre-B cell stage. *Int. Immunol.* 3:117.

25 Mayumi, M., Kuritani, T., and Cooper, M. D. 1984. T-cell regulation of IgG subclass expression by mitogen-induced plasma cells: soluble factors versus the T cells. *J. Clin. Immunol.* 4:287.

26 Beckwith, M., Urba, W., Ferris, D. K., Freier, C. E., Kuhns, D. B., Moratz, C. M., and Longo, D. L. 1991. Anti-IgM-mediated growth inhibition of a human B lymphoma cell line is independent of phosphatidylinositol turnover and protein kinase C activation and involves tyrosine phosphorylation. *J. Immunol.* 147:2411.

27 Pure, E. and Tardelli, L. 1992. Tyrosine phosphorylation is required for ligand-induced internalization of the antigen receptor on B lymphocytes. *Proc. Natl Acad. Sci. USA* 89:114.

28 Brunswick, M., Burkhardt, A., Finkelman, F., Bolen, J., and Mond, J. J. 1992. Comparison of tyrosine kinase activation by mitogenic and nonmitogenic anti-IgD antibodies. *J. Immunol.* 149:2249.

29 Ishihara, K., Wood, W. J., Damore, M., Hermanson, G. G., Wall, R., and Kincade, P. W. 1992. B29 gene products complex with immunoglobulins on B lymphocytes. *Proc. Natl Acad. Sci. USA* 15:633.

30 Matsuo, T., Kimoto, M., and Sakaguchi, N. 1991. Direct identification of the putative surface IgM receptor associated molecule encoded by murine B cell specific mb-1 gene. *J. Immunol.* 146:1584.

31 Mason, D. Y., van Noesel, C. J. M., Cordell, J. L., Comans-Bitter, W. M., Micklem, K., Tse, A. G. D., van Lier, R. A. W., and van Dongen, J. J. M. 1992. The B29 and mb-1 polypeptides are differently expressed during human B cell differentiation. *Eur. J. Immunol.* 22:2753.